Genetic Manipulation of Cell Walls

Identification of Cell Wall Traits that can be Manipulated to Improve Forage Digestibility

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Content

Introduction

The three primary roles of forages in dairy rations are to provide fiber for maintenance of healthy rumen function, energy for milk production, and protein in the case of legumes. The first two forage functions are related because it is the plant cell wall which constitutes the fiber fraction and the cell wall is of limited digestibility, thereby influencing energy availability. The forage fiber fraction also limits feed intake by dairy cows.

As genetic potential for milk production of cows is improved, their energy requirements increase. While increased feed intake can supply some of this added energy requirement, the necessity of maintaining forage in the diet for its fiber effect in the rumen limits increased feed intake potential. Therefore, to meet both the ever increasing energy requirements of high producing dairy cows, and still provide them with the fiber they require for rumen health, demands that we find ways to improve cell-wall digestibility to allow forages to contribute more energy for milk production. While post-harvest processing can be used to increase cell-wall digestibility, genetic improvements in forage digestibility are able to make lasting and more environmentally friendly gains in forage quality.

"The difficulty with improving forage quality through reduced cell-wall concentration is the fact that cell walls are needed for physical support and disease resistance of plants."

Table 1.
Variation in forage lignification.

Species	Tissue	Minimum	Maximum
ADL (% of NDF)			
Alfalfa	leaf	15.6	24.0
	stem	14.7	21.3
Corn	leaf	5.4	10.1
	stem	5.6	9.6
Klason lignin (% of cell wall)			
Corn	stem	11.8	20.8
Brome	leaf	10.8	16.3

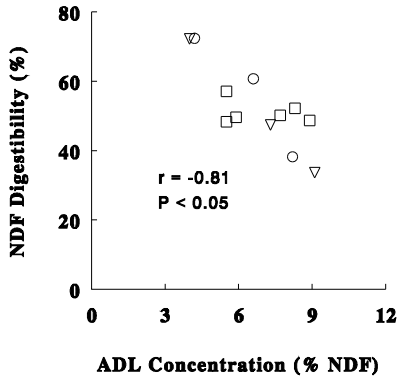


Figure 1. Relationship of lignin concentration with whole herbage in vitro 48-h NDF digestibility of smooth bromegrass (○), and switchgrass (▽) harvested at three maturity stages, and intergeneric wheatgrass hybrids (□) harvested at a single maturity (adapted from Vogel et al. 1987 and Jung and Varel 1988).

Traits of Interest

Because cell walls are of limited digestibility, selection for reduced cell-wall concentration in forages is the most obvious route to improving energy availability, because the non-cell wall components are virtually 100% digestible. Legume forages are already low in fiber if harvested at an early stage of development, therefore reduction of cell-wall concentration may not be a reasonable alternative relative to use of legumes in dairy rations. However, grasses generally contain more fiber than is needed for optimal animal performance and reduction of their cell-wall concentration may be a viable option. The difficulty with improving forage quality through reduced cell-wall concentration is the fact that cell walls are needed for physical support and disease resistance of plants. It remains to be seen how far cell-wall concentration can be reduced without adversely impacting plant viability. Buxton et al. address this point in these proceedings.

The Cell Wall Group of the US Dairy Forage Research Center has instead focused on the composition and structure of cell walls as the determining factor for digestibility. Plant cell walls are a complex matrix consisting of polysaccharides (cellulose, hemicelluloses, pectins) and lignin. Grasses also have significant concentrations of hydroxycinnamic acids (ferulate and *p*-coumarate) in their walls which we believe impact digestibility. Hatfield discusses the potential benefits of substituting pectins for the other cell-wall polysaccharides in another paper in these proceedings. In this paper we will discuss efforts to identify the components of cell-wall lignification that both negatively impact digestibility and are amenable to genetic modification through either plant breeding or molecular biology.

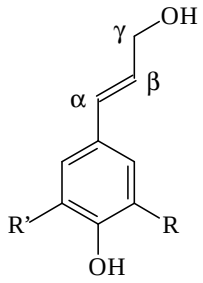
Lignin Concentration

The existence of a negative correlation between lignin concentration and forage digestibility has been known for over 50 years. For this reason, reduction of lignin concentration has been identified by numerous investigators as a goal for improvement of forage quality. Alfalfa has been selected for reduced lignin content and this approach was successful in improving total dry matter digestibility. However, further investigation revealed that by using whole herbage lignin concentration as the criterion for selection, the major result due to selection was an increase in leaf-to-stem ratio rather than a reduction in lignification of the cell wall (Kephart et al. 1989). Very little change in cell-wall digestibility was observed. This result illustrates the importance of restricting lignin selection programs to a specific plant part and to lignin as a proportion of cell-wall rather than total plant dry matter.

We have found that genetic variation in cell-wall lignin concentration exists in alfalfa, corn and smooth bromegrass (Table 1). Among a set of 190 alfalfa plant introductions significant variation existed in lignin concentration, measured as acid detergent lignin (ADL), of both the leaf and stem fractions (Jung, Sheaffer, Barnes and Halgerson unpublished). A similar result was observed by Lundvall et al. (1994) among 45 corn inbreds using the ADL method. Analysis of these same corn samples using the Klason lignin method also found significant genetic variation (Jung and Buxton 1994), although as expected the Klason lignin concentrations were greater than the corresponding ADL values. In a selection experiment with smooth bromegrass in a spaced plant nursery, Klason lignin concentrations in leaves varied widely among individual plants (Jung and Casler unpublished). These results suggest there is a large amount of genetic variation in forage species for lignin concentration that could be manipulated through plant breeding. However, a simple reduction in lignin concentration may not provide the desired increases in digestibility. Figure 1 illustrates how lignin concentration is negatively correlated with digestibility when examined across a range of maturity stages. But when the analysis is confined to plants at a single maturity, such as found in a plant breeding program, this negative relationship can vanish. For several forage species (alfalfa, birdsfoot trefoil, smooth brome-

Table 2.
Genetic variation for syringyl-to-guaiacyl ratio of lignin.

Species	Minimum	Maximum
Alfalfa	.36	.79
Corn	1.17	2.76



I Coniferyl Alcohol (R=OCH_3, R'=H)
II Sinapyl Alcohol (R=R'=OCH_3)

Table 3.
Hydroxycinnamic acids in forages.

Species	Esters	Ethers
	% CW	
Alfalfa		
p-Coumarate	0.004	0.020
Ferulate	0.003	0.099
Corn		
p-Coumarate	2.37	0.45
Ferulate	0.42	0.34

(From Jung et al. 1994, Jung and Buxton 1994)

grass, orchardgrass, switchgrass, big bluestem, and corn) we have observed no correlation, or even positive correlations, between lignin concentration and cell-wall digestibility when the influence of maturity is removed (Halim et al. 1989, Jung and Casler 1991, Jung and Russelle 1991, Jung and Vogel 1992, Jung and Buxton 1994).

As a result of these findings, we have concluded that lignin concentration is an excellent indicator of plant maturity, and because cell-wall digestibility declines with plant maturity, lignin will be negatively related to digestibility when examined across maturities. This implies that measurements of lignin concentration are of value for animal ration formulation to characterize forage quality for feeds such as hay and haylage that are harvested across a range of maturities. However, lignin concentration alone is a poor predictor of digestibility for feeds harvested at a single maturity stage (i.e. corn silage and forage breeding materials). This has led us to examine other aspects of cell-wall lignification to identify traits for modification to improve forage cell-wall digestibility.

Lignin Composition

The brown midrib (bmr) mutants identified in annual warm-season grasses, including corn and sorghum, have more digestible cell walls than their normal counterparts. These bmr mutants differ from the normal types by having less lignin, less esterified *p*-coumaric acid, and a lower syringyl content in their lignin. Because the syringyl-unit monolignol (**II**) contains a second methoxyl group compared to the guaiacyl monolignol (**I**), it has been hypothesized that syringyl-rich lignins are more linear and will penetrate a larger fraction of the cell wall and limit the wall's digestibility to a greater degree (Jung and Deetz 1993). Therefore, reduction in the syringyl-to-guaiacyl ratio of forage plant lignin was considered to be potentially beneficial to cell-wall digestibility.

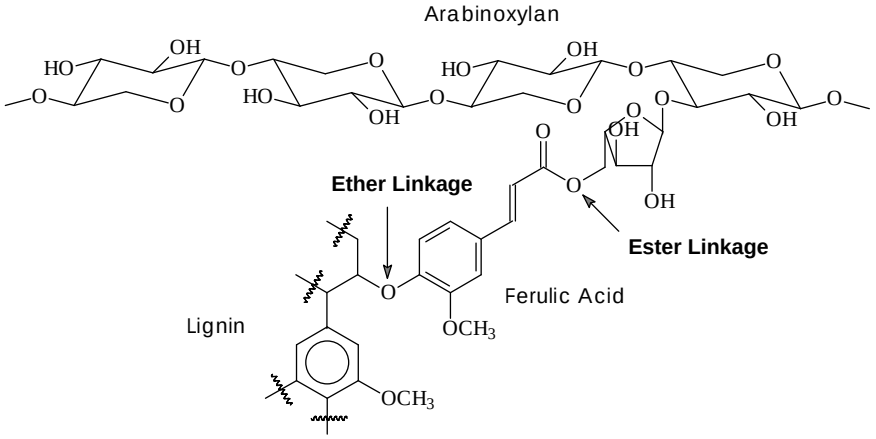
We examined 45 corn inbreds and 190 alfalfa plant introductions for genetic variation in lignin composition (Jung and Buxton 1994, Jung, Sheaffer, Barnes and Halgerson unpublished). In both species we found significant variation for the syringyl-to-guaiacyl ratio of lignin (Table 2). In neither species was lignin composition strongly correlated with lignin concentration. This implies it should be possible to reduce lignin concentration and alter lignin composition independently.

Two experiments have now been done to test the hypothesis that lignin composition will impact cell-wall digestibility. Grabber et al. (1992) compared digestibility of corn cell walls with guaiacyl- vs. syringyl-lignin polymers in their model system. At equivalent lignin concentrations the type of lignin had no effect on cell-wall digestibility. More recently we utilized a syringyl-deficit mutant of *Arabidopsis*, a species used in plant molecular biology research, to determine if lignin composition impacts cell-wall digestibility (Jung and Chapple, unpublished). Across a range of plant maturities, the mutant plants produced similar amounts of lignin compared to the controls. The mutants contained only guaiacyl- compared to normal mixed syringyl/guaiacyl-lignin, but cell-wall digestibility was the same for the mutant and control lines.

We conclude that lignin composition, at least in terms of the basic monolignol components, does not impact cell-wall digestibility. Therefore, it appears unlikely that the greater digestibility of bmr corn and sorghum is caused by their altered lignin composition. Our data also call into question the utility of current biotechnology efforts to reduce lignification by modification of the gene for *O*-methyltransferase activity if the only effect in transgenic plants is a reduction in syringyl-lignin content without reducing total lignin concentration.

Hydroxycinnamic Acids

Hydroxycinnamic acids are present in the cell walls of all forages in both ester- and ether-linked configurations, but their concentrations are much greater in grasses than legumes (Table 3). Ralph has reviewed our understanding of the hydroxycinnamic acid structures in grass cell walls in another paper in these proceedings. Here we will describe the data



III

"Cross-linking of lignin to cell-wall polysaccharides is expected to limit cell-wall digestion."

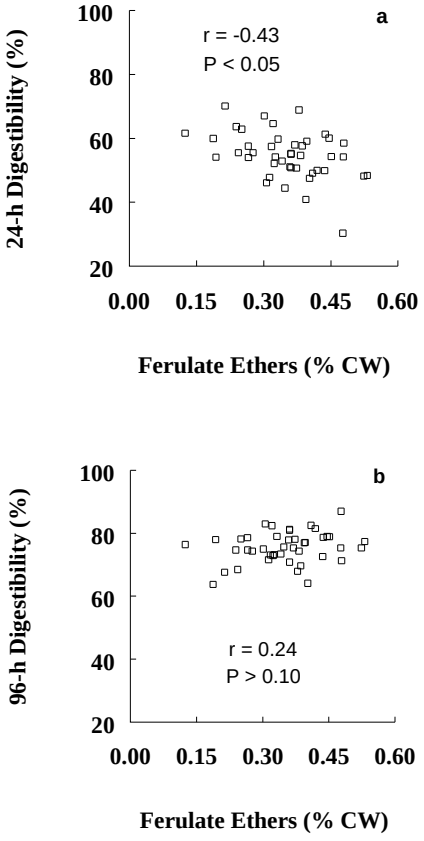


Figure 2. Relationships between corn stem internode cell-wall digestibility after 24 or 96 h fermentations and ferulate ether concentration (Adapted from Jung and Buxton 1994).

supporting our contention that ferulate cross-linking of lignin and polysaccharides in forage cell-walls reduces digestibility and our efforts to modify this inhibition.

In a study of high vs. low digestibility smooth bromegrass plants, Jung and Casler (1990) observed that the plants exhibiting the greater digestibilities had higher concentrations of esterified ferulic acid in their cell walls. It was suggested that the greater digestibility was due to lower incorporation of ferulate esters into ferulate cross-links between lignin and cell-wall polysaccharides. We knew from previous research that linkage of lignin to polysaccharide is critical for lignin to impact cell-wall digestibility (Jung and Ralph 1990). Therefore, reduction of ferulate cross-links in grass forages appears to be a reasonable target for improving cell-wall digestibility. Cross-linking of lignin to cell-wall polysaccharides is expected to limit cell-wall digestion because this bridging phenomenon is a mechanism to place the lignin in very close physical proximity to the wall polysaccharides. Because the microbial enzymes which hydrolyze the wall polysaccharides are relatively large molecules, the close proximity of lignin to the polysaccharides prevents physical access of the microbial enzymes to their substrates, thereby preventing hydrolysis (Jung and Deetz 1993). The ferulate cross-linkage of lignin and arabinoxylan (**III**) provides such a bridging and inhibitory structure. While ferulic acid molecules esterified to arabinoxylan are susceptible to removal by microbial esterases (Deetz et al. 1993), the ether linkage cannot be broken under anaerobic conditions. We expect that the ester bond in ferulate cross-links is not susceptible to enzymatic attack because the lignin polymer to which it is attached prevents the necessary microbial esterases from reaching the ester linkage. As a result the ferulate cross-link cannot be broken and maintains the close physical association of lignin and cell-wall polysaccharides. Support for this hypothesis about lack of ferulate ether degradation during digestion was found in the accumulation of ferulate ethers in the indigestible residue prepared from forages fed to sheep (Jung and Mertens unpublished).

Research with the corn cell culture model system has clearly demonstrated that reduction in ferulate cross-linking can improve digestibility under specific experimental conditions (Grabber, Ralph, and Hatfield unpublished). Direct evidence for a similar negative effect on cell-wall digestibility by ferulate cross-linking in actual forage plants has been more difficult to find. Among a set of corn inbreds, ferulate ether concentration was negatively correlated with cell-wall polysaccharide digestibility after 24-h ruminal fermentations, but a similar response was not seen after 96-h fermentations (Fig. 2). The lack of response at the longer fermentation time is puzzling as ferulate cross-linking is

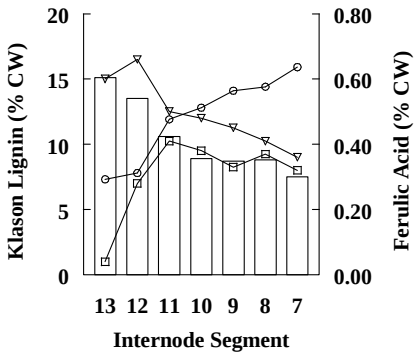


Figure 3. Changes in Klason lignin (○), and ferulate ester (▽) and ether (□) concentrations during corn stem internode development from young internodes (segment 13) to old internodes (segment 7). Bars represent the relative cell-wall polysaccharide digestibility of these cell walls.

Table 4.
Ferulate ether concentrations in grass leaves and stems.

	Maturity Stage		
Species	Veg	Boot	Head
	---------- % NDF ---------		
Leaf			
Switchgrass	0.56*	0.57*	0.43
Big Bluestem	0.39	0.42	0.44
Stem			
Switchgrass	1.10*	0.95	0.67
Big Bluestem	0.64	0.71	0.69

*Species differ within plant part ($P < 0.05$).
(From Jung and Vogel 1992)

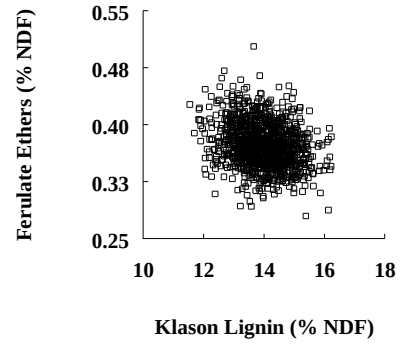


Figure 4. Variation among individual smooth bromegrass plants for leaf Klason lignin and ferulate ether concentrations.

thought to impact extent of digestion more than rate of digestion. In another study, ferulate ether concentrations of switchgrass and big bluestem were only occasionally negatively correlated with NDF digestibility for a series of comparisons within and across maturity stages and plant parts (Jung and Vogel 1992).

We have a possible explanation for why ferulate cross-linking, as measured by ferulate ether concentration, is often poorly related to cell-wall digestibility. It has been hypothesized that ferulate esters of arabinoxylan are only deposited during primary cell-wall growth of plants (Jung and Deetz 1993). Ralph et al. (1995) have now demonstrated that monolignol units, rather than pre-formed lignin polymer, are etherified to the ferulate ester during lignification, strongly suggesting that ferulate esters are the initiation or nucleation sites at which lignin polymerization begins. If ferulic acid is only deposited during early cell-wall development and then the esters are incorporated into the cross-links with lignin, then ferulate ester and ether concentrations will decline during maturation because of continued deposition of lignin and polysaccharides while no further ferulic acid is deposited in the wall. This dilution effect could easily obscure the impact of ferulate cross-linking on digestibility when regression analyses are based purely on concentration.

Recent research results obtained on the development of cell walls in corn stem internodes provides support for the preceding hypothesis (Morrison, Jung, Buxton and Hatfield unpublished). We found that ferulate ester concentrations were high in cell walls of young internodes and then declined with maturation (Fig. 3). At the same time ferulate ethers rose sharply during early development of internodes, but then also declined as lignin deposition continued. This pattern agrees with our proposed scheme of ferulic acid deposition and cross-linkage formation in grass cell walls. We also noted that ferulate ether concentrations were strongly correlated to cell-wall polysaccharide digestibility during early stages of development, before the dilution effects of later wall growth exerted their influence. These results indicate that selection for improved cell-wall digestibility by reducing ferulate cross-linking will need to be done on young tissues to avoid the confounding factors that arise later during development.

We have documented species differences for ferulate ether concentration between switchgrass and big bluestem, and identified genetic variation among corn inbreds and within smooth bromegrass for ferulate cross-linking. Switchgrass and big bluestem differ in ferulate cross-linking at early, but not later, stages of reproductive development (Table 4). Among 45 corn inbreds, ferulate ether concentration in the stem ranged from below detection limits (~ 0.01%) to 0.53% of the cell wall (Jung and Buxton 1994). Similarly, individual smooth bromegrass plants exhibited ferulate ether concentrations that ranged from 0.28 to 0.50% of NDF (Jung and Casler unpublished). Clearly variation exists between and within most, if not all, grass species for ferulate cross-linking of lignin and cell-wall polysaccharides.

A selection study is in progress with smooth bromegrass to select plants with high and low concentrations of Klason lignin and ferulate ether cross-links in young leaf tissue. Our aim is to test the relative impact of lignin vs. cross-linking on forage intake and cell-wall digestibility. Figure 4 illustrates the variation we observed among 1100 individual plants (Jung and Casler unpublished). While the variation appears large and suggests that breeding for reduced ferulate cross-linking should be possible, there are two important negative factors that must be considered. First, development of a rapid, NIRS based screening method has not been successful. The NIRS calibrations are poor and we have had to depend on laborious wet-chemistry analysis of ferulate ethers. Second, initial indications are that the genotype x environment interaction is large for both ferulate ether and Klason lignin concentrations. This has necessitated a multiple year evaluation of individual plants before making selections for further development. We are, however, continuing this project and hope to

identify some genetically divergent plants for ferulate cross-linking by next spring to use in an animal study evaluating cross-linking's effects on cell-wall digestibility and intake.

We have also embarked on a molecular biology based approach to reducing ferulate cross-linking. Our goal is to identify the gene which attaches ferulic acid to arabinoxylan via the ester linkage. We chose to target this step in cross-linkage biosynthesis because it occurs intra-cellularly and should be under strict enzymatic control, whereas the etherification step of cross-linkage formation is driven by a free-radical reaction in the cell-wall space and is probably under less strict enzymatic control. Transposon mutagenesis was used to generate mutations in corn plants. Over 10,000 corn seedlings from these lines have been screened for impaired ferulate ester synthesis. After this initial screening and subsequent testing we have identified two plants which are low in ferulate ester concentration (two standard deviations or more below the mean) and this reduction in ferulate esters is inherited by their selfed progeny (Ni, Jung and Phillips, unpublished). The transposable element is being used to isolate mutated genes from these corn plants, and DNA fragments are currently being sequenced and evaluated.

Conclusion

We believe that improving cell-wall digestibility through genetic modification of the forages is ultimately the most effective method for improving energy availability from forages for dairy cattle. We have chosen to concentrate on specific components of the cell wall because other research groups, including some in industry, are using selection for NDF digestibility. The US Dairy Forage Research Center's Cell Wall Group is uniquely qualified to pursue this more basic approach. We have identified cell-wall traits that should, and should not, be targets for manipulation, and we have demonstrated that genetic variation to utilize in selection does exist for these traits in economically important forages.

Analysis of the relationships between cell-wall lignification and digestibility has reinforced the fact that the cell wall is a complex structure with many interactions. A series of our studies have found that the inclusion of several aspects of lignification (i.e. lignin concentration and composition, hydroxycinnamic acids and their linkage forms) are generally required to predict forage cell-wall digestibility when maturity is not a factor (Jung and Vogel 1992, Jung et al. 1994, Jung and Buxton 1994, Deetz et al. 1996). Even then there is still a large amount of the variation in cell-wall digestibility that cannot be explained by our chemical analyses. Wilson and Mertens (1995) have recently proposed that cellular anatomy and organization of cells in the plant may limit access to potentially digestible cell walls by rumen microbes. This new hypothesis could explain why chemical constituents of forage cell walls have not completely explained the rate and extent of wall digestion.

The Cell Wall Group has initiated several experiments to evaluate the impact of accessibility on digestibility. We will combine this new information with our knowledge of cell-wall composition and structure to more fully explain the limits to digestion as we strive to improve forage energy availability to dairy cows.

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